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GM-CSF Induces STAT5 Binding at Epigenetic Regulatory Sites within the *Csf2* Promoter of Nonobese Diabetic (NOD) Mouse Myeloid Cells

F. Seydel^{1,2,3}, E. Garrigan^{1,2}, B. Stuttevoss¹, N. Belkin¹, B. Makadia¹, J. Carter¹, J.-D. Shi⁴, A. Davoodi-Semiroomi^{1,5}, M. McDuffie⁶, and S. A. Litherland^{1,7}

¹ Dept of Pathology, Immunology, and Laboratory Medicine, University of Florida, 100275 JHMHC, 1600 SW Archer Rd., University of Florida, Gainesville, Florida 32610

⁴ Dept of Biochemistry and Molecular Biology, College of Medicine, University of Florida, 100275 JHMHC, 1600 SW Archer Rd., University of Florida, Gainesville, Florida 32610

⁶ College of Medicine, University of Virginia, Charlottesville, VA

Abstract

Myeloid cells from nonobese diabetic (NOD) mouse and human type 1 diabetic (T1D) patients overexpress granulocyte-macrophage colony stimulation factor (GM-CSF). This overproduction prolongs the activation of signal transduction and activator of transcription 5 (STAT5) proteins, involved in GM-CSF-induced control of myeloid cell gene expression. We found that GM-CSF can regulate the binding of STAT5 on the promoter of its own gene, *Csf2*, within regions previously identified as sites of chromatin epigenetic modification important to the regulation of GM-CSF during myeloid differentiation and inflammation. We found multiple sequence polymorphisms within NOD mouse chromosome 11 *Idd4.3* diabetes susceptibility region that alter STAT5 GAS binding sequences within the *Csf2* promoter. STAT5 binding at these sites *in vivo* is increased significantly in GM-CSF-stimulated-bone marrow cells and in unactivated, high GM-CSF-producing macrophages from NOD mice as compared to non-autoimmune C57BL/6 mouse myeloid cells. Thus, GM-CSF overproduction by NOD myeloid cells may be perpetuating a positive epigenetic regulatory feedback on its own gene expression through its induction of STAT5 binding to its promoter. These findings suggest that aberrant STAT5 binding at epigenetic regulatory sites may contribute directly to immunopathology through cytokine-induced gene expression dysregulation that can derail myeloid differentiation and increase inflammatory responsiveness.

Keywords

autoimmunity; cytokines; diabetes; monocyte/macrophage; signal transduction

⁷corresponding and senior author: S. A. Litherland, PhD, Assistant Professor, Dept of Pathology, Immunology, and Laboratory Medicine, College of Medicine, University of Florida; current address: Research Associate Professor, Burnham Institute for Medical Research-Lake Nona, SLSL Bldg M6-1025, Kennedy Space Center, FL 32899; Phone 321-861-2016; Fax 321-861-2226; email-sal@burnham.org.

²These authors contributed equally to these studies.

³Current address: College Ave, Livermore, CA

⁵Current address: College of Medicine, University of Central Florida, Orlando, FL

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Introduction

Granulocyte-macrophage colony stimulating factor (GM-CSF) promotes the differentiation of bone marrow myeloid precursor cells into granulocytes, monocytes, macrophages and dendritic cells [1–5]. After differentiation, GM-CSF acts as an integral activation signal for monocytes and macrophages in inflammatory responses [6,7]. Expression of GM-CSF is tightly regulated during the temporal sequence of cytokines promoting normal non-autoimmune myeloid cell hematopoiesis, with its effects in myeloid cell differentiation are predominantly felt before the influence of macrophage colony stimulating factor (M-CSF) and granulocyte colony stimulating factor (G-CSF). Following commitment to the myeloid lineage, cell responsiveness to GM-CSF must be suppressed for these subsequent lineage specific cytokines (G-CSF, M-CSF) to complete the maturation process [1]. If not, cells will progress only to intermediate phenotypes or suffer elimination in the process [1,4,5]. After the exposure to M-CSF in differentiation, mature monocytes and macrophages can again respond to GM-CSF, though now as an activation, not a differentiation stimulant [6,7].

In inflammation, mature myeloid cells produce GM-CSF in response to toll-like receptor (TLR)-induced activation, IL-6, and IL-1 β cytokine signaling [8,9]. Within the first 4–6hr of the inflammatory response, GM-CSF supports the activation of prostaglandin synthase 2/cyclooxygenase 2 (PGS2/COX2), the dual function enzyme involved in early response pro-inflammatory prostanoid production [6,10]. Equally important, GM-CSF also induces IL-10 production and responsiveness in the cells approximately 10hr after the initial insult, allowing for resolution of inflammation and a return to homeostasis [1,11]. Such tight temporal regulation of both expression and responsiveness to GM-CSF during differentiation and inflammation relies heavily on cytokine-induced epigenetic control of chromatin upstream of its coding region, through histone acetylation/deacetylation modifications [4,8,9]. Several sequence specific sites in the *Csf2* promoter have been described as association sites for the histone deacetylase, SMRT/NCoR, in response to IL-6, IL-1 β , and IL-2 signaling in myeloid cells and T cells [8,9].

Myeloid antigen presenting cells (APC) in both humans with autoimmune type 1 diabetes (T1D) and the nonobese diabetic (NOD) mouse spontaneously overexpress GM-CSF [12,13]. Autoimmune myeloid cell overproduction of GM-CSF induces persistent activation of the signal transduction/transcriptional regulatory proteins, STAT5A and STAT5B [13,14], which act as secondary signal molecules as well as adaptor proteins epigenetic histone modification enzymes [15,16].

Myeloid APC dysfunction in autoimmunity has been linked to defects in cytokine signaling during differentiation and chronic inflammation [17–23]. Myeloid APC differentiation is disrupted in the NOD mouse by defective M-CSF signaling that is not linked to a lack of expression of either M-CSF or its receptor in these cells, but rather to a lack of responsiveness at the intracellular signaling level [20]. Since M-CSF also regulates STAT5 activation in its effects on myeloid cell gene expression [24,25], we hypothesized that the excessive GM-CSF stimulation of these signaling proteins may be disrupting subsequent response of APC to M-CSF.

In T1D human monocytes and NOD macrophages, GM-CSF-activated STAT5 proteins exhibit aberrant DNA binding and subcellular localization [13]. Congenic mouse analysis of NOD chromosome 11 revealed that these STAT5 and GM-CSF traits are linked to NOD polymorphisms in the *Idd4.3* diabetes susceptibility region of this chromosome, along with the majority of NOD diabetes susceptibility conferred by this region [14]. The *Idd4.3* locus contains the gene for GM-CSF, *Csf2*, along with several other cytokines that use STAT5 in

their signaling, but excludes the genes for STAT5A and STAT5B from participating in its effects [14,26].

Since STAT5 proteins can associate with SMRT/NCoR to promote histone deacetylation as well as with CBP/P300 acetylase for histone acetylation [15,16,27], we set out to determine whether GM-CSF could induce STAT5 binding at its own promoter at the sites previously found to associate with these chromatin modification enzymes; and thereby, possibly autoregulate its own gene's expression through STAT5-SMRT/NCoR or STAT5-CBP/P300 complexes. Our findings indicate that GM-CSF regulates STAT5 binding to DNA in the non-coding sequence upstream of *Csf2*, the gene encoding for GM-CSF, primarily in regions previously reported as epigenetic chromatin modification sites within the *Csf2* promoter [8, 9]. Sequence analysis revealed multiple polymorphisms in NOD DNA with the *Csf2* promoter, affecting at least 2 STAT5 binding sites. Using anti-tyrosine phosphorylated STAT5 (STAT5Ptyr)-mediated chromatin immunoprecipitation (ChIP) and realtime PCR analysis, we found that GM-CSF stimulated STAT5 binding at these sites in both NOD immature bone marrow cells and mature macrophages, while decreasing it in these myeloid cell populations in a non-autoimmune mouse strain, C57BL/6. These data suggest that GM-CSF-induced STAT5 DNA binding can affect its own *Csf2* gene's expression, presumably through facilitating epigenetic regulation; and thus, may be the underlying cause of both the overexpression of GM-CSF and the prolonged activation of STAT5 seen in autoimmune monocytes and macrophages. This derailment of GM-CSF-mediated gene regulation could have grave consequences for both myeloid differentiation and control of inflammation as implicated in the immunopathogenesis of autoimmune diabetes.

Methods

Mouse Strains

Five to twelve week old NOD and C57BL/6 female mice (The Jackson Laboratory, Bar Harbor, ME) were used for all studies. At least 2 mice of each strain were used for tissues in each run of the experiment and each analysis was run at minimum in triplicate. These strains were maintained as breeding stock in the University of Florida College of Medicine Pathology Department SPF colony, in microisolator cages with food and water without restriction. All procedures were conducted according to IACUC approved protocols B083 and D754.

Bone Marrow In vitro Differentiation & Sample Preparation

Mice were euthanized and the long bones of the hind limbs were excised. Bone marrow cells were flushed out of the bones using a 30-gauge needle and syringe filled with ice cold RPMI medium supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic mix (Cellgro-Mediatech, Herndon, VA). The marrow cells were washed with cold media then the red blood cells in samples were lysed by incubation in sterile cold 0.84% NH₄Cl buffer. The remaining bone marrow cells were then plated on tissue culture dishes and fed with fresh, sterile medium alone or with 1000U/ml of GM-CSF (Biosource/Invitrogen, Carlsbad, CA) then followed 30min after with the addition or omission of 100μM Na vanadate in DMSO (Sigma-Aldrich, St Louis, MO) to the culture medium. Cultures were maintained for 2 (with and without Na vanadate) or 24hr (without Na vanadate) at 37C/5%CO₂. An aliquot of cells was taken to confirm phenotypic identification and phosphotyrosine STAT5 analysis by flow cytometry as previously described [13,14]. After incubation, half of the media volume from these cultures was collected and frozen at -80C to confirm GM-CSF concentration by Luminex (Upstate Biotech Beadlyte kits, Upstate USA, Millipore, Chicago, IL) and ELISA (BD Biosciences OptEIA kits, San Diego, CA) as previously described [12-14]. Cells were fixed *in situ* with 1%(v/v C_f) formaldehyde (methanol-free, Sigma-Aldrich) added in the remaining media for 10min at 37C, then washed with 1xPBS, and sonicated in SDS Lysis Buffer (Upstate) + protease

inhibitors (Roche, Indianapolis, IN) for later analysis in chromatin immunoprecipitation (ChIP).

Monocyte and Macrophage Collection & Culture

Mice were euthanized and approximate 50 μ l of blood collected via cardiac puncture post mortem. Blood was immediately processed for analysis of STAT5 phosphorylation by flow cytometry as previously described for NOD myeloid cells [14]. After blood collection, the peritoneal cavity was filled with ice cold RPMI medium supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic mix (Cellgro-Mediatech, Herndon, VA) using a 20-gauge needle and syringe. The lavage fluid and cells were withdrawn and washed with cold media by centrifugation. Liver tissue was collected after lavage for use in genomic DNA preparation. Red blood cells in lavage samples were lysed by incubation in sterile cold 0.84% NH₄Cl buffer. The remaining cells were then plated on tissue culture dishes for 1 hour at 37C/5% CO₂. The non-adherent cells were washed off the plates with sterile 1XPBS (Cellgro-Mediatech) and fed the adherent macrophage re-fed with fresh sterile medium alone or with 1000U/ml of GM-CSF (Biosource/Invitrogen) for future *ex vivo* culture at 37C/5% CO₂. An aliquot of cells was taken to confirm phenotypic identification and phosphotyrosine STAT5 analysis by flow cytometry as previously described [13,14]. After incubation, half of the media volume from these cultures was collected and frozen at -80C to confirm GM-CSF concentration by Luminex (Upstate) and ELISA (BD Biosciences) as previously described [12-14]. Cells were fixed *in situ* with 1%(v/v C_f) formaldehyde (methanol-free, Sigma-Aldrich) added in the remaining media for 10min at 37C, then washed with 1xPBS, and sonicated in SDS Lysis Buffer (Upstate) + protease inhibitors (Roche) for later analysis in chromatin immunoprecipitation (ChIP).

Sequence Analysis of Csf2 Gene Promoter

Genomic DNA from each strain was prepared from liver and amplified in PCR using Roche Biosciences Master Mix reagents and primers (3'-3bp: CTA AAG CAT GTT TCT TGG CTA; 3'-350bp: AGA AGC AGT TCC TGA TTC CA; 3'-642bp: AAA GAG GCT CAC ATA ACT CA; 5'-969bp: AAA TAA GGT CCA GCC CAA TG; Integrated DNA Technologies, Coralville, IA) designed to amplify regions in the -3 to -969bp sequence upstream of the *Csf2* gene. The amplified DNA was gel purified using Qiagen gel extraction reagents and phenol/chloroform extracted (Qiagen, Valencia, CA). The amplified fragment was then used as template in a Big Dye PCR amplification reaction (Applied Biosystems) and sequenced using an AB capillary sequence analyzer (Applied Biosystems). ChromasLite and ClustalW freeware were used for the sequence analysis and alignment. Analysis of secondary structure in amplicon primers and sequence regions was analyzed using Oligo 4.0 primer analysis software copyrighted by Wojciech Rychlik.

Chromatin Immunoprecipitation (ChIP) Analysis of STAT5 Binding at the Csf2 Promoter

Four million cells from bone marrow cultures or *ex vivo* peritoneal macrophages were fixed *in situ* with 1% formaldehyde in 1xPBS (methanol-free, Sigma-Aldrich) for 10min at 37C prior to lysis with High SDS Lysis Buffer (Upstate). The fixed lysates were sonicated to disrupt membranes and shear chromatin to approximately 1000bp fragments then frozen until analysis. Once thawed, the samples were divided into aliquots for each run of the analysis. The aliquots used for IP were pre-cleared with salmon sperm DNA Protein A or G agarose beads (Upstate), then incubated overnight at 4C with anti-tyrosine phosphorylated STAT5 (STAT5Ptyr) antibodies (Upstate). After incubation, the antibody-bound chromatin complexes were precipitated using salmon sperm DNA Protein A agarose beads, and washed extensively with a series of increasing stringency buffers (low salt, high salt, LiCl, TE; ChIP reagent kit, Upstate). A non-specific antibody control (mouse IgG, UpState) and a no extract sham IP were run as negative controls.

Total cell and ChIP extract aliquots were dissociated from the beads in 1% SDS, 0.1M Bicarbonate buffer (Fisher Scientific, Atlanta, GA). NaCl was then added to a final concentration of 500mM and the samples incubated 4hr at 65C to reverse the formaldehyde crosslinks. DNA was purified from these aliquots for PCR and real time PCR amplification of DNA sequences from *Csf2* promoter which have been shown to be epigenetic regulatory sites for inducible *Csf2* expression [8,9].

ChIP isolated DNA samples were volume matched in all PCR runs to 100ng of total DNA extracts from same samples. Real time PCR was run using Sybr Green Master Mixes (Applied Biosystems, Foster City, CA or BioRad, Hercules, CA) in a 98C hot-start protocol designed to remove secondary structure in the DNA template. Due to the pallindromic nature of STAT5 binding sites, DNA containing these regions forms complex secondary structure which can block polymerization *in vitro*, we modified our PCR protocol to include the addition of 2%-5% DMSO (Sigma-Aldrich) in the reaction mix and a cycle profile of 98C 5min, 94C 30 sec, 55–60C (dependent on the primer set used) 30sec, 72C 30sec, for 45 cycles. Real time amplification quantitation was compared on the basis of R values calculated as $R = \frac{2^{(\text{Nonspecific Ig ChIP Ct}) - (\text{anti-STAT5Pty ChIP Ct})}}{2^{(\text{Nonspecific Ig ChIP Ct}) - (\text{anti-STAT5Pty ChIP Ct})}}$ [28]. Statistical analyses of data were performed using Prism 4 software (GraphPad, San Diego, CA).

Results

Since GM-CSF plays pivotal roles in gene regulation for both myeloid differentiation and mature myeloid cell contributions to the inflammatory process, we examined its expression in both immature bone marrow precursor cells and in mature peritoneal macrophages from the autoimmune diabetic NOD mice and from non-autoimmune C57BL/6 mice. As we previously reported for NOD macrophage and monocytes [12–14], NOD bone marrow cells have increased GM-CSF expression and high STAT5 phosphorylation compared to C57BL/6 mouse bone marrow cells (Figure 1). However, the STAT5 phosphorylation in NOD bone marrow cells was significantly lower than in more mature cells, despite its relatively high (i.e., not significantly different than mature cell) GM-CSF production. This may reflect the lower percentage of specific myeloid precursor cell in the mixed cell population of bone marrow or may indicate a more sensitive GM-CSF responsiveness or the development of GM-CSF independent persistence of STAT5 activation in more mature cells[12,13]. Deconvolution image analysis of GM-CSF and M-CSF *in vitro* differentiated NOD bone marrow cells suggest the former rather than the latter is the most likely explanation (Maton-Rumore et al, in submission).

NOD *Csf2* Promoter Region contains a unique microsatellite DNA insertion

Previous congenic mouse analysis of chromosome 11 linked NOD phenotypes in STAT5 phosphorylation, DNA binding, and, GM-CSF production all with *Idd4.3*, and not the downstream *Stat5a/Stat5b* loci [14]. Since the *Idd4.3* locus contained *Csf2*, the gene encoding GM-CSF, we postulated that these phenotypes could be functionally linked to a GM-CSF-responsive STAT5 binding sites within the *Csf2* promoter. Comparative sequence analysis of the region 1kb upstream of the *Csf2* gene in NOD and non-autoimmune C57BL/6 mice revealed a microsatellite DNA insertion in the NOD not found in the control strain (Figure 2). This 1kb region also contained at least 2 potential STAT protein GAS (gamma activation sequences) binding sites (TTCNNNGAA/AAGNNNCTT) [29,30], in both 5' to 3' and 3' to 5' orientations. There are also several 'half' or imperfect STAT5 binding sites within this region. Such suboptimal sites have the potential for binding STAT proteins as part of multimeric binding facilitated by STAT protein bound at a nearby GAS site [30,31]. At least 2 potential STAT5 binding sites (boxed in Figure 2b) within this region are altered in the NOD as compared with C57BL/6; one lost (5'->3') and one gained (3'->5'). At the 3' to 5' site, C57BL/6 mice have a

GAS site that can bind either STAT6 or STAT5, with preference to STAT6; whereas, the NOD sequence at this region contains a strong STAT5 binding site that would not support STAT6 binding [29–31].

Increased STAT5 Binding on the *Csf2* Gene Promoter in NOD Macrophages & Bone Marrow Cells

To test for the possible involvement of STAT5 in regulation of the GM-CSF gene *Csf2* itself, we performed STAT5Ptyr-mediated ChIP to isolate STAT5-associated chromatin and then identified *Csf2* promoter DNA by PCR using primers designed to detect identified deacetylase binding sites within the first 1000bp of the promoter region upstream of the *Csf2* gene coding sequence [8,9]. STAT5 proteins in NOD peritoneal macrophages exhibit strong binding on sequences immediately upstream of the *Csf2* gene (−181bp to +10bp [8]), without exogenous GM-CSF stimulation; whereas, STAT5 binding at this site was not found in C57BL/6 cells (Figure 3). There was also a marked increase in the presence of *Csf2* promoter DNA in anti-STAT5Ptyr-mediated ChIP isolates from untreated NOD bone marrow cells compared to those of C57BL/6 (Figure 4a).

When STAT5 binding within the entire −3 to −969bp region upstream of the *Csf2* transcriptional start site was analyzed in bone marrow cells, DNA from anti-STAT5Ptyr bound complexes from both NOD and C57BL/6 showed an overall decrease in STAT5 binding after GM-CSF stimulation compared with untreated cultures (Figure 4a). However, STAT5 binding at identified epigenetic regulatory regions tested showed a marked enhancement with GM-CSF stimulation in NOD bone marrow cells not seen in the C57BL/6 (Figure 4a&b). These findings suggested that GM-CSF is enhancing STAT5 binding only at specific regulatory sites within its own gene's promoter in NOD, while reducing it at all sites in C57BL/6 bone marrow cells. Analysis of individual epigenetic regulatory sites within the *Csf2* promoter indicates that GM-CSF increases STAT5 binding at each site tested but to varying degrees (Figure 4b), but does not define whether STAT5 binding at each site is independent, or synergistic to each of the other sites.

Discussion

Chronic inflammation and defective myeloid differentiation are common phenotypes associated with more than one autoimmune disease and thought to play a central role in the loss of APC tolerogenic functions contributing to immunopathology [2,20,23]. Our previous studies have shown that GM-CSF expression in autoimmune myeloid cells is aberrantly high and promotes activation of the epigenetic enzyme adaptor proteins, STAT5A and STAT5B [12,13]. Congenic analysis of Chromosome 11 from NOD mice eliminated the possible direct involvement of any STAT gene to these myeloid cell phenotypes, and implicated the genetic region containing the gene encoding GM-CS, *Csf2*, in both GM-CSF overexpression and STAT5 overactivation [14].

Chen et al[9] and Ito et al[8] have shown that the *Csf2* gene, encoding the myeloid cell differentiation and activation cytokine GM-CSF, is regulated by chromatin epigenetic modification, and that this regulation is important to controlling the inflammatory response of monocytes and macrophages, both by other cytokines and pharmacologically. Therefore, we examined the potential for GM-CSF to promote its own expression in autoimmune myeloid cells through epigenetic modification associated with a positive feedback loop of STAT5 DNA binding and functioning as a histone acetylase/deacetylase adaptor protein.

In our previous *in vitro* binding analyses [13], we showed that even brief exposure to GM-CSF promoted STAT5 binding to *Ptg2* enhancer and *Cis* promoter DNA *GAS* sequences in bone marrow derived myeloid cells from non-autoimmune mice but not from NOD mice. In contrast,

GM-CSF exposure enhanced and prolonged DNA binding of activated STAT5 at these sites from NOD macrophages compared to cells from non-autoimmune mice. Although we could isolate an abundance of phosphorylated truncated STAT5 isoforms from the cytoplasm of NOD bone marrow-derived myeloid cells, these proteins were unable to bind DNA *in vitro* even with prolonged GM-CSF stimulation [13]. We originally thought this discrepancy in STAT5 binding in *in vitro* DNA binding assays could be due to the presence of stage-specific truncated STAT5 isoforms found in abundance in immature NOD myeloid cells.

Truncated STAT5 isoforms were first thought to be naturally occurring variants created by post-translational modification by a nuclear serine protease [3]. These truncated STAT5 isoforms lack their carboxy termini; and therefore, have lost their ability to bind the histone acetylase, CBP/P300 [3,15,16,27]. Truncated STAT5 proteins could facilitate gene repression because they can still be adaptor molecules for the histone deacetylase, SMRT/NCoR [15]. However, recent studies have suggested that cathepsin G released during *in vitro* extraction processing may be responsible for STAT5 truncation [32,33]. These findings have thrown the physiological significance of these isoforms into doubt.

Furthermore, Neculai et al [34] have suggested that the tyrosine phosphorylation sites of STAT proteins are more accessible to phosphatase removal when STAT proteins in dimer conformations that can bind DNA, and less accessible on those in non-binding conformations. These new studies indicated that our previous *in vitro* binding data, which relied heavily on the subcellular extraction methods to differentiate STAT5 molecules capable of binding chromatin in the nucleus, may have been skewed towards detecting the majority of STAT5 dimers which were found in non-DNA binding conformations that collected in the cytoplasm over time, and which would be more easily accessible for Cathepsin G cleavage. In addition, STAT5 complexes capable of binding DNA in these cells could have been lost to our analysis due to their increased sensitivity to dephosphorylation.

In light of these reports, we re-evaluated our *in vitro* data, and determined that since STAT5 functions *in vivo* to bind chromatin not naked DNA, it is conceivable that synthetic binding sites used in these analyses may have been suboptimal substrates for STAT5. Thus, these analyses were inadequate for detection of the lower level of activated full-length STAT5 present in the bone marrow cells compared to macrophages.

To improve our detection of STAT5 binding capacity in this study, we switched to the more sensitive and accurate ChIP *in vivo* binding analysis. Using ChIP, we found that both NOD and C57BL/6 STAT5 DNA binding in bone marrow cells but that GM-CSF enhanced specific full-length STAT5 binding at the *Csf2* promoter site only in NOD mouse bone marrow cells but not in the non-autoimmune C57BL/6 mouse cells. GM-CSF induced detectable full-length STAT5 binding on sites within the *Csf2* promoter. These data suggest reflect GM-CSF's ability to regulate its own expression through a positive feedback loop mediated through STAT5. Such a regulatory mechanism would have the potential to increase histone acetylation within the *Csf2* promoter, through the recruitment of CBP/P300, resulting in epigenetic dysregulation of this chromosomal region, and promote the overexpression of GM-CSF seen in NOD myeloid cells. Western blot analyses of STAT5Ptyr-precipitated chromatin-protein complexes suggest that GM-CSF activation of STAT5 in NOD myeloid cells promotes interactions of STAT5 with chromatin along with CBP/300, and increased acetylated histone H3, but have not yielded data that suggest SMRT/NCoR is found in these complexes (Rumore-Maton et al and Garrigan et al, manuscripts in submission).

It is possible that temporal window for controlling GM-CSF's influence on myeloid differentiation and in inflammatory responses may be overridden as a consequence of prolonged GM-CSF production in autoimmune cells. The work of Hashimoto et al [1] indicates

that loss of this window of GM-CSF independence in myeloid differentiation leads to loss of subsequent responsiveness to M-CSF, IL-10 and precipitates inadequate myeloid APC maturation, similar to the myeloid differentiation dysfunction seen in the NOD [20].

The mouse *Csf2* promoter region has an overall 61% homology with human *Csf2* promoter, and even higher homology in the specific epigenetic regulatory regions focused on in these studies [8,9]. Thus, polymorphisms within the *Csf2* promoter may provide evidence for a genetic component underlying the aberrant GM-CSF and STAT5 phenotypes which NOD myeloid cells share with human autoimmune myeloid cells. We are further analyzing the *Csf2* promoter region in congenic NOD mouse strains to narrow the interval(s) involved in changes in STAT5 binding, epigenetic modification, *Csf2* gene dysregulation in myeloid APC, and diabetes immunopathology. These results will allow us to test these sequences in enhancer/promoter reporter constructs to solidify their functions in *Csf2* expression. Preliminary data from these studies support the genetic link to *Csf2* expression and further suggest that STAT5 binding to epigenetic regulatory sites within the *Csf2* promoter impacts not only GM-CSF expression, but GM-CSF induction of persistent STAT5 activation and STAT5-mediated dysregulation of *Ptgs2* [Garrigan et al, manuscript in preparation]. Thus, the dysregulation of *Csf2* may be indicative of more widespread effects on the expression of other genes regulated by GM-CSF through STAT5.

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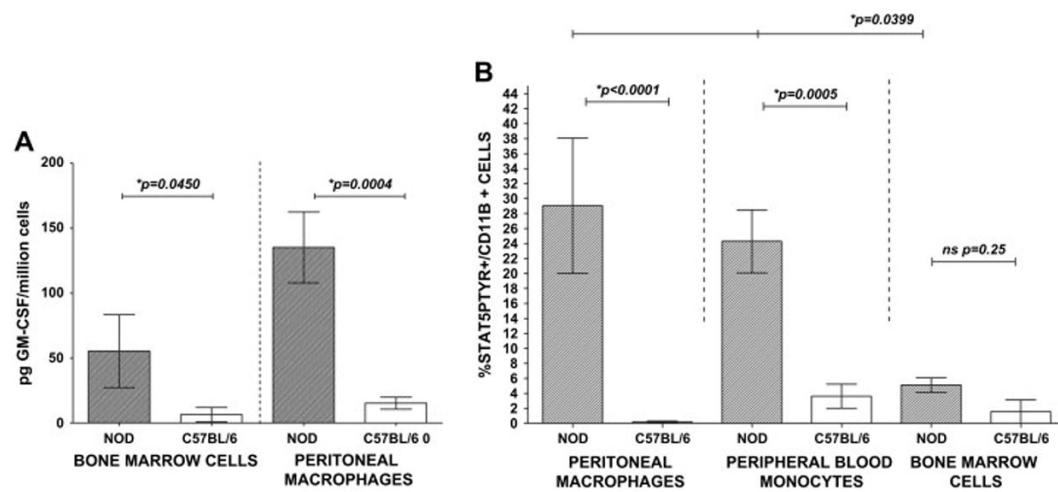


Figure 1. GM-CSF Production and STAT5 Phosphorylation are Aberrantly High in NOD Mouse Myeloid Cells

A. Four to five million bone marrow cells and adherence-isolated peritoneal macrophages were cultured without supplementation for 24hr at 37°C/5%CO₂. Cell-free culture supernatants were then analyzed by ELISA and/or Luminex for the presence of GM-CSF. GM-CSF concentrations were normalized to pg/million plated cells for comparison. The p values listed were obtained from Mann-Whitney U test analysis of the data. Patterned bars indicate the mean GM-CSF production from NOD samples and open bars the mean of C57BL/6 samples. Error bars represent SEM. **B.** *Ex vivo* myeloid cells from NOD and C57BL/6 mice (peritoneal macrophages, peripheral blood, and bone marrow cells) were collected and fixed within 4hr of collection and then analyzed for phosphorylated STAT5 by intracellular flow cytometry [12–14]. Data shown represents the percentage of gated CD11b⁺ cells that are also positive for phosphorylated STAT5. In macrophage and bone marrow cell analyses, 10000 events were counted, while 5000 events were collected for each run of the peripheral blood analysis. The p values listed were obtained from Mann-Whitney U test analysis of the data. Patterned bars indicate the mean %STAT5Ptyr⁺/CD11b⁺ cells detected in NOD samples and open bars the mean of C57BL/6 samples. Error bars represent SEM. The p values listed are from pair wise (Student t or Mann Whitney U) or group wise ANOVA analyses.

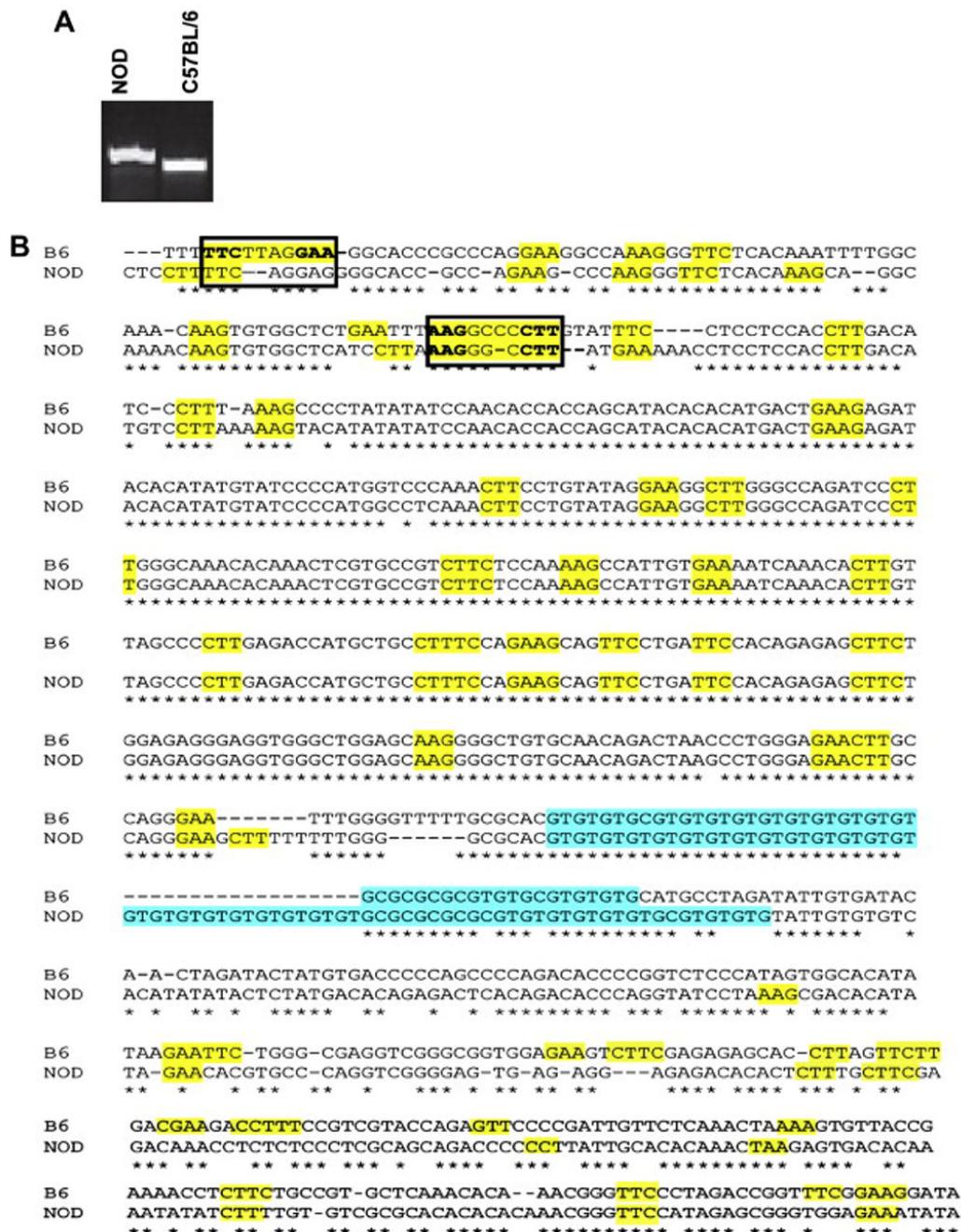


Figure 2. Sequence Comparisons for *Csf2* Promoter Region Reveals Polymorphism in NOD
A. Genomic DNA from NOD and C57BL/6 mice were amplified in PCR using primers specific for the -181 to +10bp region upstream of the *Csf2* gene [9]. PCR products run on a 5% agarose gel show a small but reproducible size variation between the two mouse strains. Data are representative of 12 runs of the analysis. **B.** Sequence analysis of the -3 to -969bp region upstream of the *Csf2* gene in NOD and C57BL/6 mouse genomic DNA samples revealed differences in STAT5 binding sites (yellow, boxed), many half or imperfect binding sites (yellow), and a microsatellite DNA insertion (blue) in the region that has a length polymorphism between the two strains. Putative STAT5 binding site NOD polymorphisms are

indicated by underlined bold, boxed sequences. * indicate sequence homology between the two strains.

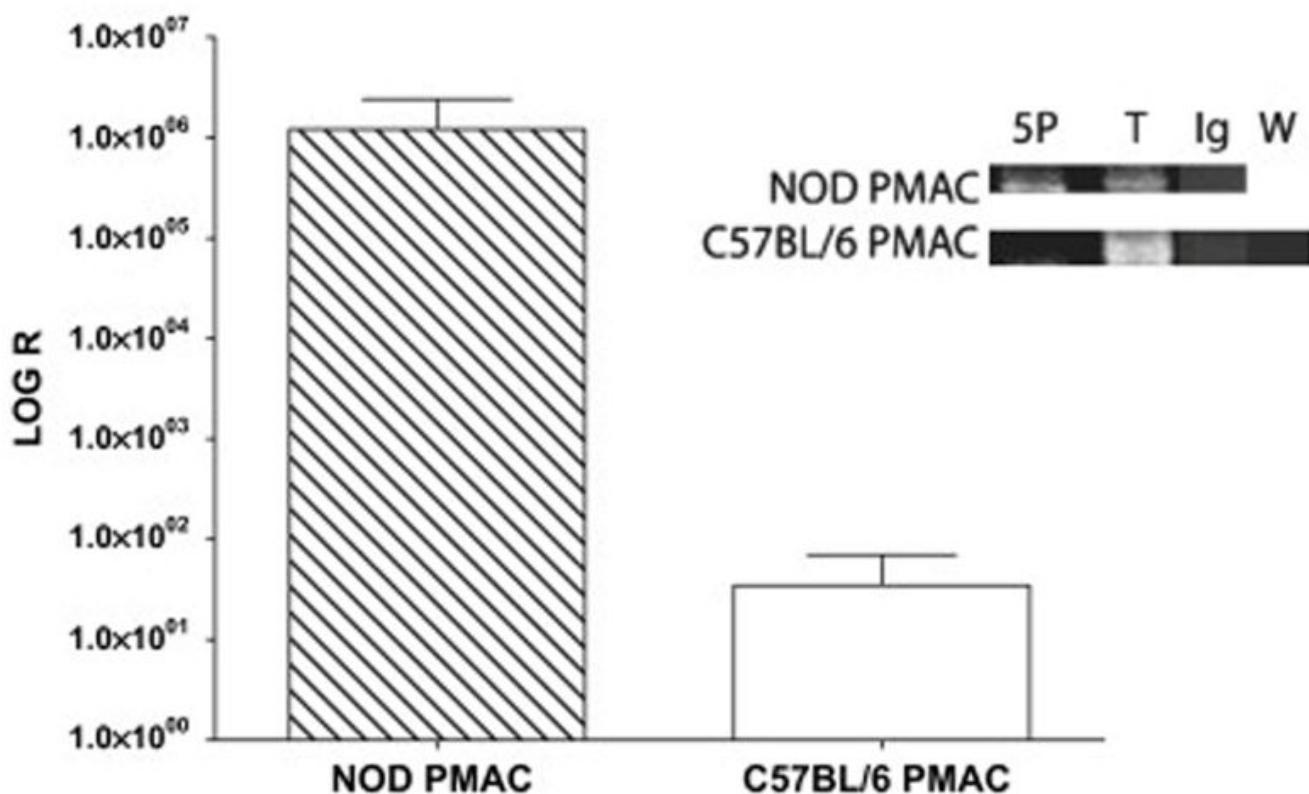


Figure 3. Macrophage Chromatin Immunoprecipitation (ChIP) Analysis shows STAT5 binding within the promoter region upstream of the gene which encodes for GM-CSF, *Csf2*
 Four million peritoneal macrophages (PMAC) from NOD and non-autoimmune C57BL/6 mice were incubated for 24hr without exogenous GM-CSF. Cells were fixed *in situ*, and extracted for protein-chromatin complexes as described in the Materials & Methods. Extracts divided in 4 parts and immunoprecipitated with anti-STAT5Ptyr antibodies. One hundred nanograms of the ChIP-isolated STAT5Ptyr-associated DNA were analyzed by conventional PCR/Ethidium bromide agarose gel (insert) and by SybrGreen Real Time PCR for the presence of *Csf2* promoter (-181 to $+10$ bp) DNA sequences. Key: 5P= ChIP anti-STAT5 precipitated DNA, T= total cellular DNA from unprecipitated fixed cell extracts, Ig= ChIP non-specific mouse IgG precipitated DNA, W= DNA-free water control. Patterned bars (log of mean R values) and gel represent data obtained from 3 independent runs of each strain. Error bars represent SEM.

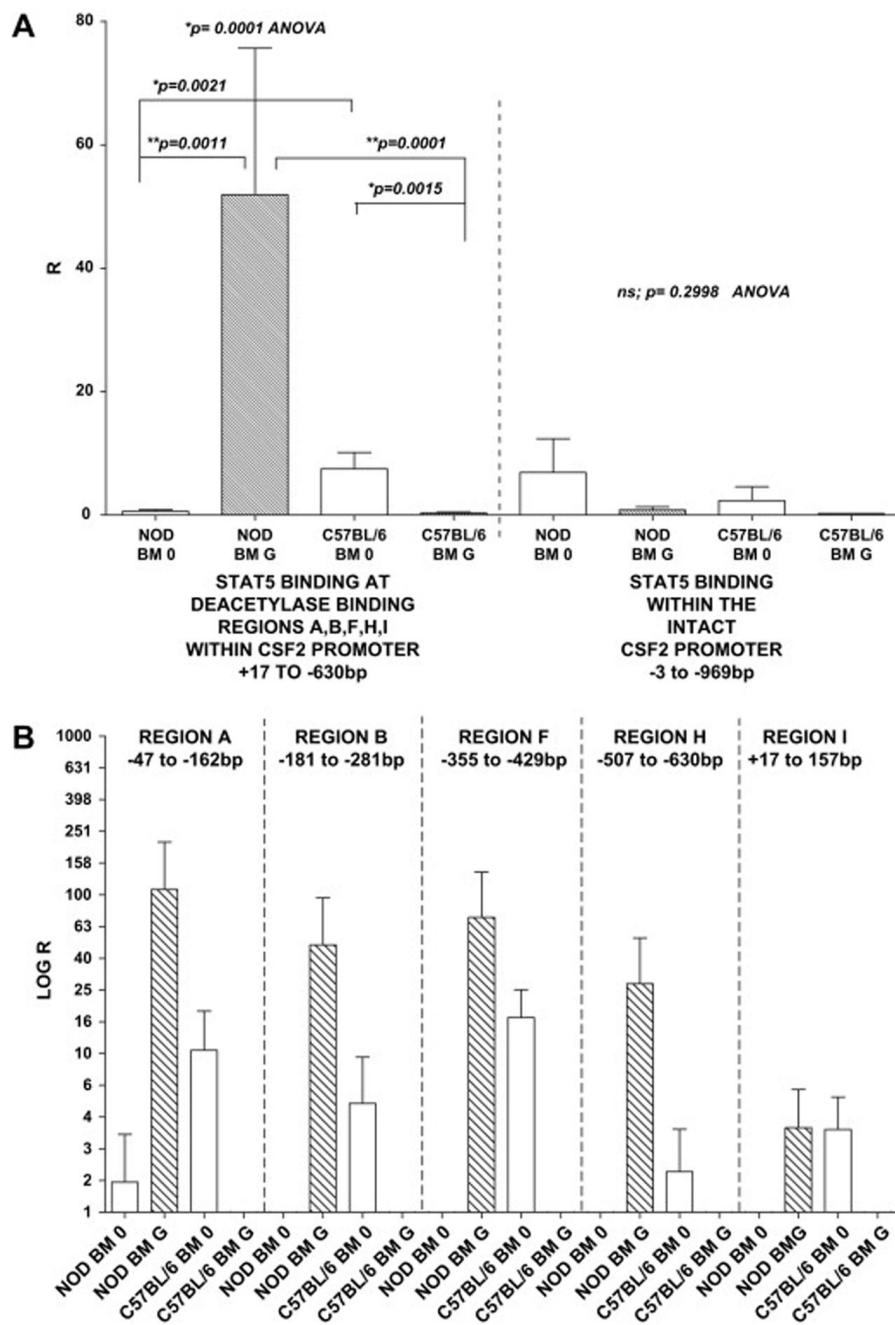


Figure 4. ChIP Analysis of GM-CSF-Induced STAT5 Binding Upstream at Multiple sites within the *Csf2* Promoter involves DNA Secondary Structure

Four million cell cultures of **NOD** and **C56BL/6** mouse bone marrow cells (**BM**) were grown for 24hr in the presence (**GM** or **G**) or absence (**0**) of 1000U/ml GM-CSF before being fixed and extracted for ChIP analysis. Aliquots of 100ng of total DNA extracted from ChIP protein-chromatin complexes precipitated with anti-STAT5 antibodies were amplified using primers to potential epigenetic modification sites within the *Csf2* promoter region [8,9]. **A.** Real time PCR analysis using specific primers to amplify and identify *Csf2* promoter regions previously identified as epigenetic control sites for *Csf2* gene expression. Unpatterned bars indicate cells without treatment (**0**) and hatched bars indicate cells treated with GM-CSF (**G**). Data

representative of 2–3 sample sets. **B.** Real time PCR analysis using the hot start and DMSO protocol described in the Materials and Methods to remove secondary structure and the primers depicted in **A** to amplify and identify *Csf2* promoter regions previously identified as epigenetic control sites for *Csf2* gene expression. Unpatterned bars indicate cells without treatment (**0**) and hatched bars indicate cells treated with GM-CSF (**G**). Data representative of 2 (combined A–I) and 3 (Promoter –3 to –969bp) sample sets. The p values indicate one-way ANOVA analysis (above graphs) or Mann-Whitney U tests (on graph) of pairwise comparisons.